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Results: HbS, HbC, HbE, Hb South Florida and Hb Ullevaal show typical and distinct patterns in the globin LC/MS according to the theoretical protein data. The tryptic digest of Hb Ullevaal resulted in the identification of the respective mutated peptide βT9, which was confirmed by genetic sequencing.
Conclusions: By the application of globin-LC/MS two more dimensions for the Hb identification are added, hydropathicity and protein mass. With this workflow as screening procedure for Hb variants it is expected to be able to detect and identify the majority of variants with the exception of highly unstable variants, which cannot be determined in the peripheral blood at all. A negative result makes the presence of a significant Hb variant in the peripheral blood improbable.

Keywords: globin differentiation; Hb Ullevaal; hemoglobin variants; mass spectrometry; tryptic digest.

Zusammenfassung

Hintergrund: Ungefähr 2/3 der Hämoglobin-Varianten weisen keine Ladungsdifferenz zum Wildtyp auf, aber die meisten davon unterscheiden sich in der Hydrophobizität. Ergänzend zur Kationenaustauscher-Chromatografie wurde die Globin-Differenzierung mittels LC-Tandem-MS eingeführt. Die Untersuchung von Hb Ullevaal wurde als Beispiel gewählt, da zu dieser Hb-Variante bisher noch keine Protein- oder Peptid-Daten publiziert wurden.
Methoden: Das Screening auf Hb-Varianten wird mittels Kationenaustauscher-HPLC durchgeführt. Die Globin-Trennung erfolgte mittels RP-LC/MS. Nach dem Peptidverdau mit Trypsin wurden die Peptide mittels reversed phase-HPLC und anschließender Tandem-MS detektiert. Es erfolgte eine Datenbanksuche für die Identifizierung mutierter Fragmente. Für die Bestätigung des Hb Ullevaal wurde die Sequenzierung des β-Globin-Genes durchgeführt.
Ergebnisse: Für HbS, HbC, HbE, Hb South Florida und Hb Ullevaal zeigten sich typische, den theoretischen Proteindaten entsprechende Muster in der Globin-LC/MS. Mittels tryptischem Verdau konnte das mutierte Peptid von Hb

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Ullevaal identifiziert werden. Hb Ullevaal wurde durch Sequenzierung des β-Globin-Genes bestätigt.
Schlussfolgerungen: Durch die Einführung der Globin-LC/MS stehen mit der Hydrophobizität und der Protein-Masse zwei weitere Dimensionen für die Hämoglobin-Identifizierung zur Verfügung. Mittels dieses Vorgehens ist zu erwarten, dass ein Großteil der Hb-Varianten detektiert und identifiziert werden können. Eine Ausnahme bilden hyperinstabile Hb-Varianten, die im peripheren Blut nicht nachweisbar sind. Ein negatives Ergebnis macht das Vorliegen einer signifikanten Hb-Variante im peripheren Blut sehr unwahrscheinlich.

Schlüsselwörter: Globin-Differenzierung; Hämoglobin-Varianten; Hb Ullevaal; Massenspektrometrie; Trypsin-Verdau.

Introduction

A total of 1268 genetically determined variants of hemoglobin (Hb) have been reported to date [1]. Although about 26% of the variants already characterized have been shown to influence the physiologic behavior of the Hb, others do not seem to have an influence or are still not sufficiently characterized [1]. Often variants are discovered accidentally during determination of glycated hemoglobin (HbA_{1c}). Either the variant itself or a post-translational modified entity may interfere with the correct determination of HbA_{1c} independent whether the determination is performed by chromatography or with antibody based assays [2–5]. Antibody based HbA_{1c} assays are principally not able to detect variants. Even variants which are prevalent in several populations have a direct or indirect influence on the measured HbA_{1c} what makes it desirable to gain knowledge about their presence [6, 7].

Notwithstanding the genetic testing is considered to be the gold standard for the detection of Hb variants neither comparably higher costs nor the necessary efforts justify the use of it as a first line screening procedure. While about 2/3 of the variants do not show a charge difference to the wildtype entity, >95% of them differ in hydrophobicity (Figure 1) [8]. This implies that screening procedures relying only on differences of the isoelectric point probably will be insensitive to 2/3 of possible variants. Beside this it recently has been shown that phenotype data can be indispensable for a comprehensive identification of an Hb variant [9]. Hb Ullevaal was chosen as an example because the first description is based only on genetic data while no further experimental data at the protein level have been published [10].

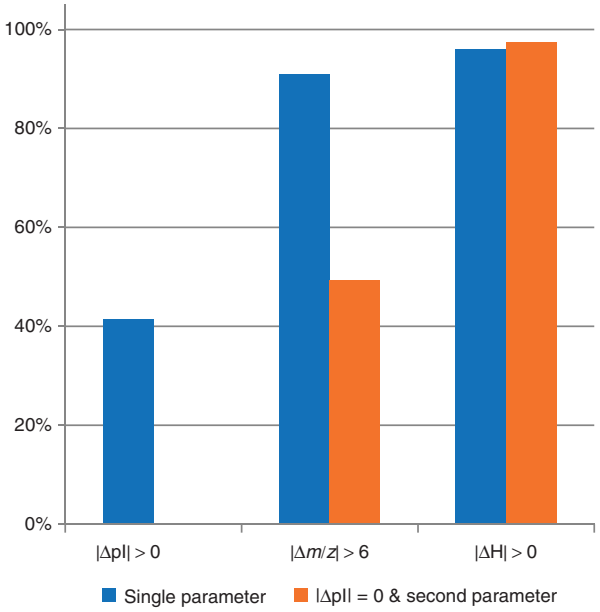

Figure 1: Percentage of variants of α- and β-globin with a single parameter differing from wild type (blue) and percentage of variants with no charge difference (ΔpI), but differing mass (ΔM) or differing hydropathicity (ΔH) (orange).

Materials and methods

Patients

The patient sample described here for Hb variant determination has been drawn from a 78-year-old German male which showed an interfering peak in the HbA_{1c} determination by ion exchange HPLC (Figure 2). The informed consent of the patient was obtained.

For all procedures described here with K_3-EDTA anticoagulated blood samples were used.

Several examples of the globin separation of abnormal Hbs are obtained from anticoagulated routine samples for which Hb diagnostic was requested.

Materials

Tissue culture grade trypsin from porcine pancreas, calcium chloride, N-p-Tosyl-L-phenylalanine chloromethyl ketone (TPCK), dithiothreitol (DTT), iodoacetamide and trifluoro acetic acid (TFA) were purchased from Sigma-Aldrich, Munich, Germany. Tris(hydroxymethyl) methylamine and sodium chloride were obtained from Merck, Darmstadt, Germany. LCMS-grade methanol and acetonitrile were purchased from J.T. Baker, Netherlands. LCMS-grade water was used for all applications and was obtained from a Milli-Q Reference water purification system, Merck Millipore, Darmstadt, Germany.

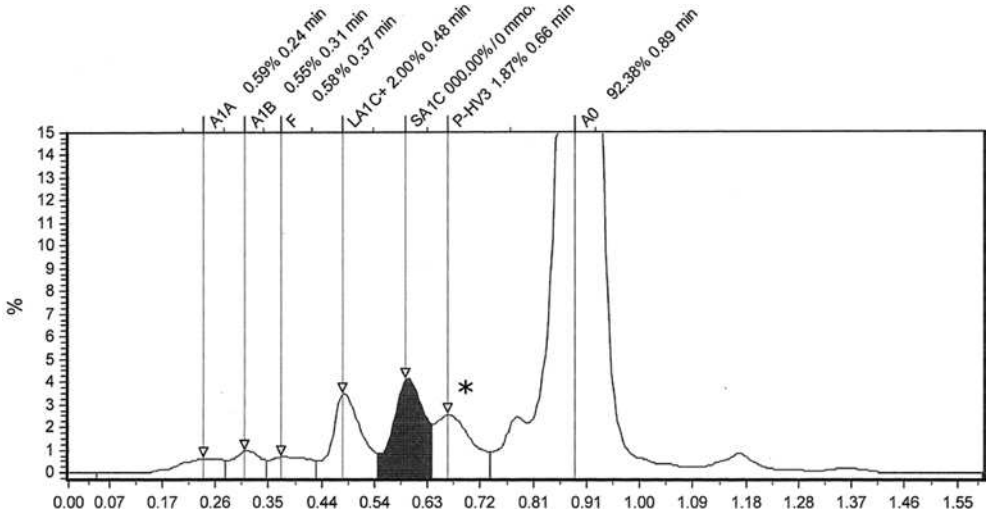

Figure 2: Chromatogram of HbA_{1c} determination of Hb Ullevaal with Tosoh G8, Variant mode.
The grey shaded peak represents HbA_{1c}. The peak (*) probably represents glycated Hb Ullevaal or another derivative causing an interference hindering the integration algorithm to correctly determine the HbA_{1c}.

Methods

The blood count (Sysmex-2100 XE, Kobe, Japan) and a cation exchange separation of the intact Hb (Tosoh G8, Tokyo, Japan, thalassemia mode) have been performed according to the instructions of the manufacturer.

A stock solution of trypsin was prepared in aliquots of 1 mg/mL in 1 mmol/L hydrochloric acid stabilized with 20 mmol calcium chloride and stored at −80 °C. Stock solutions of 0.9% sodium chloride in water, 0.5 mol/L Tris-HCl (pH 8), 50 mmol/L DTT in water, 100 mmol/L iodoacetamide in water and 10 mmol/L TPCK in methanol have been prepared.

For sample preparation K_3-EDTA-anticoagulated blood samples were centrifuged 5 min at 10,000 *g* at ambient temperature, the plasma was removed, the red blood cells one time washed with saline at 4 °C and for hemolysis the five-fold amount of water at 4 °C was added, the sample vortexed and centrifuged at 20,000 *g*/10 min. The supernatant was adjusted with water to a Hb content of about 10 mg/mL.

The tryptic digest was performed with 25 µL of the hemolysed sample by adding 5 µL Tris-buffer and 3.5 µL DTT-solution at 37 °C for 30 min, 3.85 µL iodoacetamide solution at 37 °C for 30 min, 10 µL DTT at 37 °C for 30 min and 5 µL acetonitrile, 0.5 µL TPCK-solution and 5 µL trypsin solution for 20 h at 37 °C. The digest was stopped by adding 5 µL TFA.

The liquid chromatography mass spectrometry was performed on a Thermo Vantage system consisting of an Ultimate UHPLC and TSQ Vantage mass spectrometer (Thermo Fisher Scientific, Dreieich, Germany) used with positive ESI. The globin separation was performed on a RP-column (Aeris Widebore 2.1×250 mm, Phenomenex, Torrance, USA) using water/TFA 0.1% (mobile phase A) and acetonitrile/TFA 0.1% (mobile phase B) as gradient (39% B–46% B in 15 min and 46% B–100% B in 1 min, flow rate 0.5 mL/min).

The separation of the tryptic peptides was performed on a reversed phase-column (Aeris Peptide 2.1×250 mm, Phenomenex) using water/0.1% formic acid (mobile phase A) and methanol/0.1% formic acid (mobile phase B) as linear gradient (5% B–25% B in 30 min and to 60% B–100% B in 10 min).

As parameters for the HESI ion source in the positive mode a spray voltage of 3000 volt, vaporizer temperature of 550 °C, sheath gas pressure setting 50, auxiliary gas setting 20 and a capillary temperature of 250 °C have been used.

The scan parameters for the intact globin scan were set to full scan 750–1500 *m/z* with a mass resolution width setting of 0.2 *m/z* and a scan time of 0.75 s for routine globin analysis. Tryptic peptides are first analyzed by a survey scan with 220–1500 *m/z* using a mass resolution width setting of 0.5 *m/z* and a scan time of 0.5 s. As next step under the same chromatographic conditions data dependent scans are performed with 1 mTorr argon as collision gas to gain fragment ion spectra of the respective tryptic peptides. Alternatively targeted fragment ion spectra are produced from the formerly identified aberrant doubly charged peptides.

Proteomic data analysis

The globin mass was derived by spectra deconvolution of the multiply charged globin ions (charge state 11+ to 20+) [11]. For mass calibration of the algorithm, the α-globin spectrum with nominal *m/z* of 15126.3 of the uncharged globin was used as *m/z* reference.

In the tryptic digest, the mutation bearing peptide was identified by comparing the survey scan of the patient sample with a wild type control sample. The chromatograms are checked for new appearing peaks and corresponding peaks with reduced intensity compared to the wild type sample. As first validation step, the mass difference of the putative mutated peptide and the corresponding wild type peptide has to be in accordance with the formerly estimated mass difference of the intact globins.

For identification of tryptic peptides a FASTA file of wild type and single point mutated α- and β-globins and the tryptic peptides to be expected was prepared. Raw spectral fragmentation data of the respective peptide were merged. For identification of the tryptic peptides The GPM X!Tandem [12] was used to fit the spectra against the

previously prepared FASTA library. The result then has to be graphically validated.

Genetic analysis

For single cases of HbS, HbE and Hb South Florida and Hb Ullevaal the mutations by genetic sequencing have been confirmed as well (data not shown). Exon 1 + 2 were amplified with primers DHPLC1 Forward (5′-GCA ATT TGT ACT GAT GGT ATG G-3′) and DHPLC3 Reverse (5′-CCA CAC TGA TGC AAT CAT TCG-3′) and exon 3 with primers DHPLC4 Forward (5′-CAT CAG TGT GGA AGT CTC AGG-3′) and HPLC7 Reverse (5′-TTA AAT GCA CTG ACC TCC CAC-3′) using Accustart II PCR master mix according to manufacturer's instructions.

Sequencing was done with the same primers at Microsynth. Sequences were analyzed by aligning them to HBB Refseq NG_000007.3.

Results

Hematological investigations of the patient sample bearing Hb Ullevaal showed a macrocytosis and hyperchromia of the red blood cells (Ery count 4.4 T/L, Hb 14.8 g/dL, MCH 34 pg, HCT 44.3%, MCV 100 fl, RDW 12.9%, MCHC 33 g/dL). This is different from the already published case in which the results have been within the reference range [10]. ALT and γ-glutamyl transferase are elevated, but lactate dehydrogenase, cobalamin and folic acid were found in the reference range. However, neither holotranscobalamine nor methylmalonic acid have been determined so that a pernicious anemia cannot be excluded as cause for the macrocytosis. The elevated liver enzymes point to the possible presence of other pathologies. The blood sample was submitted for determination of glycated Hb, which showed a fraction interfering with the glycated Hb and prevented correct peak integration (Figure 2).

Ion exchange HPLC

The cation exchange separation of intact Hb on the Tosoh G8 represents a basic technique requiring a charge difference of the respective variant. Common wide spread variants (HbS, HbE, HbC) easily can be discovered and quantified by this method. The studied patient sample containing Hb Ullevaal does not show any abnormality in this ion exchange chromatography (Figure 3).

Globin-RP-LC/MS

For the patient sample a distinct extra peak in the globin separation was visible representing about 50% of the β-globin, thus pointing to a heterozygous β-globin variant. The deconvolution of the multiply charged globin spectrum yields a single mass peak of 15,854 *m/z* which corresponds to a difference of –14 *m/z* compared to the wild type β chain (Figure 4). Only 40 of 861 possible single point mutations of β-globin are expected to show this pattern [8].

Tryptic digest

In the peptide map in comparison with a wild type sample an additional peptide was detected, at the same time

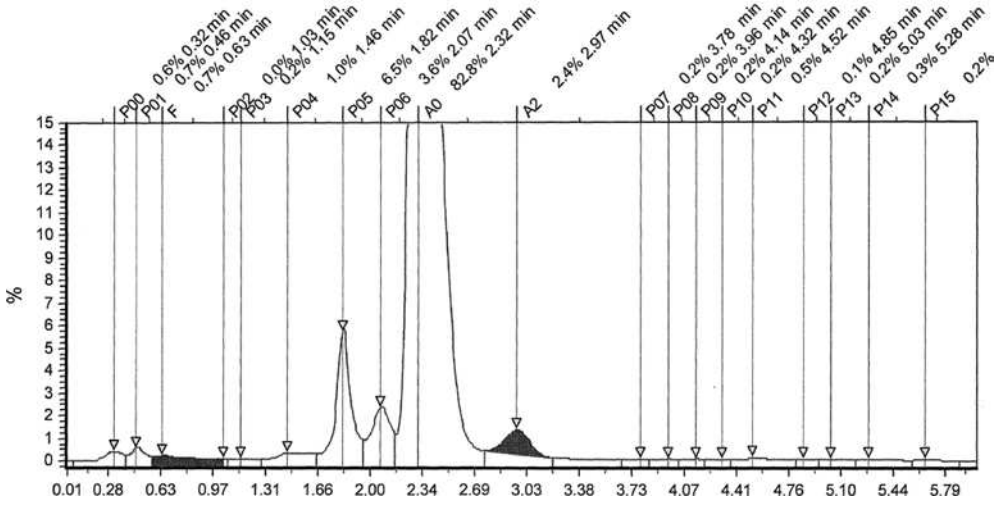

Figure 3: Cation exchange chromatogram of heterozygous Hb Ullevaal (Tosoh G8, thalassemia mode). This represents a normal finding without any suspicious fraction.

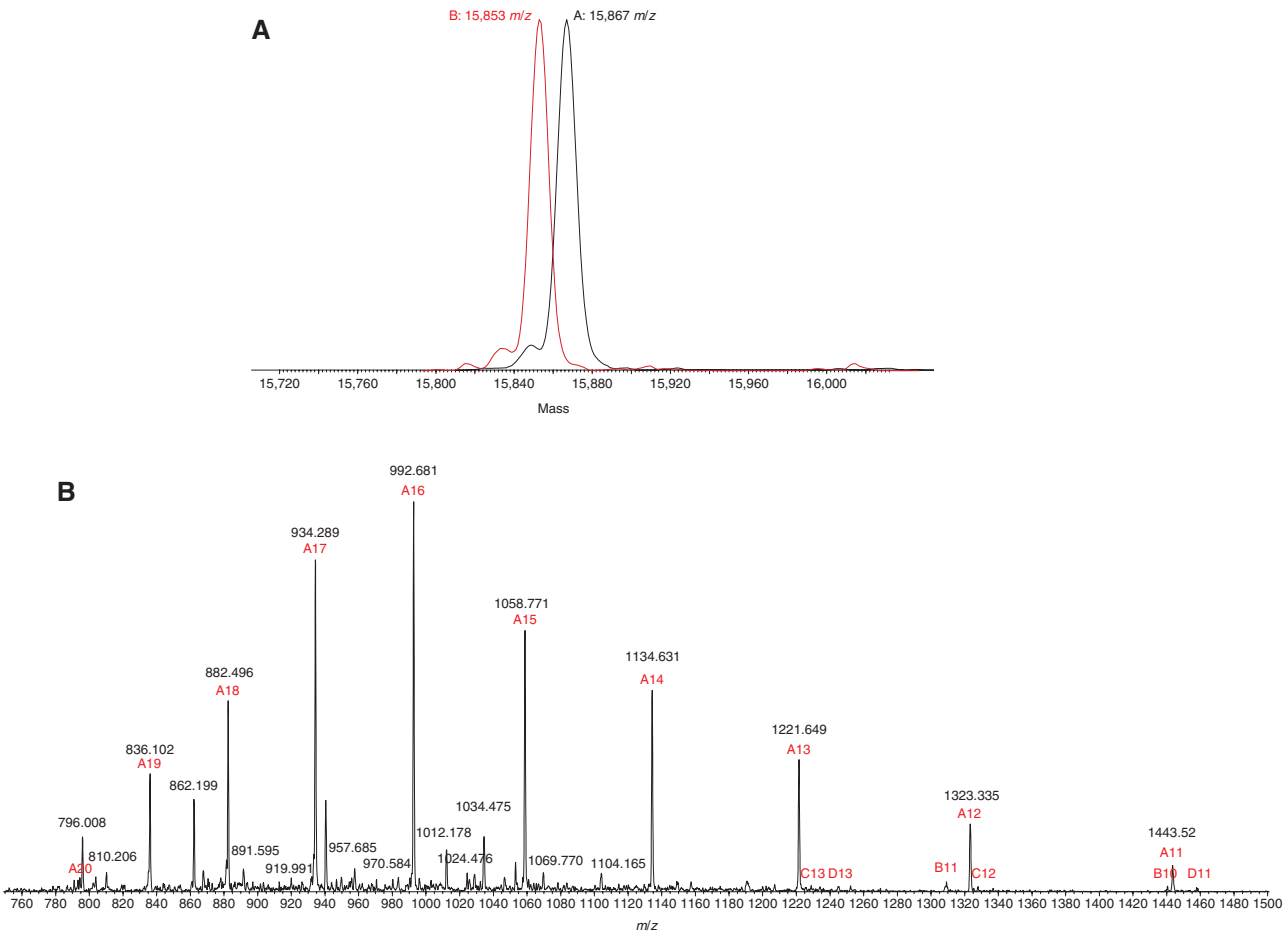

Figure 4: A: Deconvolution results of the β globin ESI+ mass spectrum.
(A) Wild type, (B) Hb Ullevaal (red). B: Spectrum of the multiply charged β-globin.

another peptide shows a markedly reduced intensity (Figure 5). The difference in the peptide mass proofed to be –14 *m/z*, hence it is identical with the mass difference of the undigested globins. The wild type peptide was measured to have a mass of 1668.5 *m/z*, corresponding to βT9. In this tryptic peptide 7 mutations are expected to show similar characteristics.

For the final identification a product ion scan of the doubly charged aberrant peptide was performed. Fifteen product ion spectra have been obtained, which are used for identification with the X!Tandem algorithm of *The GPM*. This unequivocally resulted in the identification of Hb Ullevaal (Figure 6), other database hits are not found. Furthermore, if the prepared globin-mutation-fragment database was not included in the search, not any hit was found within all available human and other databases.

Thus, the exchange β78(EF2)Leu→Val has been confirmed.

Genetic testing

Sequencing of the β-globin gene detected a heterozygote C→G transition of the first nucleotide of codon 78. This CTG→GTC transition leads to the Leu→Val exchange in the β-chain as described above. The variant was thus identified as Hb Ullevaal also by DNA sequencing.

Discussion

Until today, separation techniques relying on a charge difference are the main basis for screening if Hb variants are suspected. This includes chromatographic or electrophoretic procedures like capillary zone electrophoresis. Although this criterion is important and brings along the detection of the Hb variants most frequently found, the larger part of possible Hb variants are not expected to show

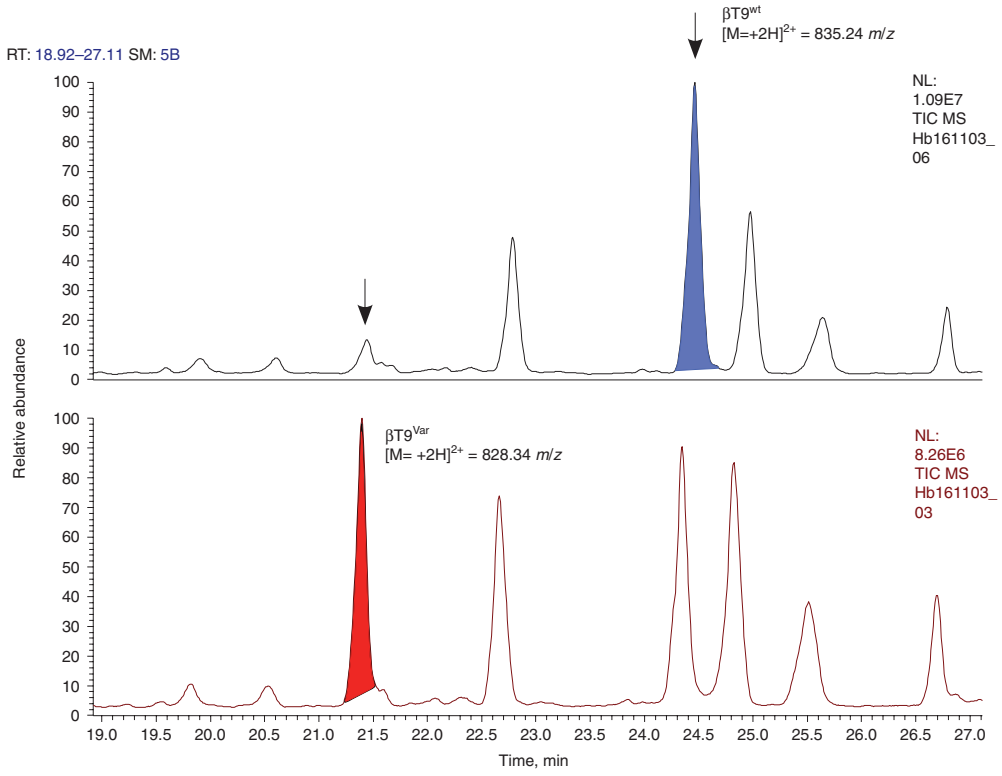

Figure 5: Peptide map showing βT9 wild type (wt, blue) and Hb Ullevaal (Var, red).

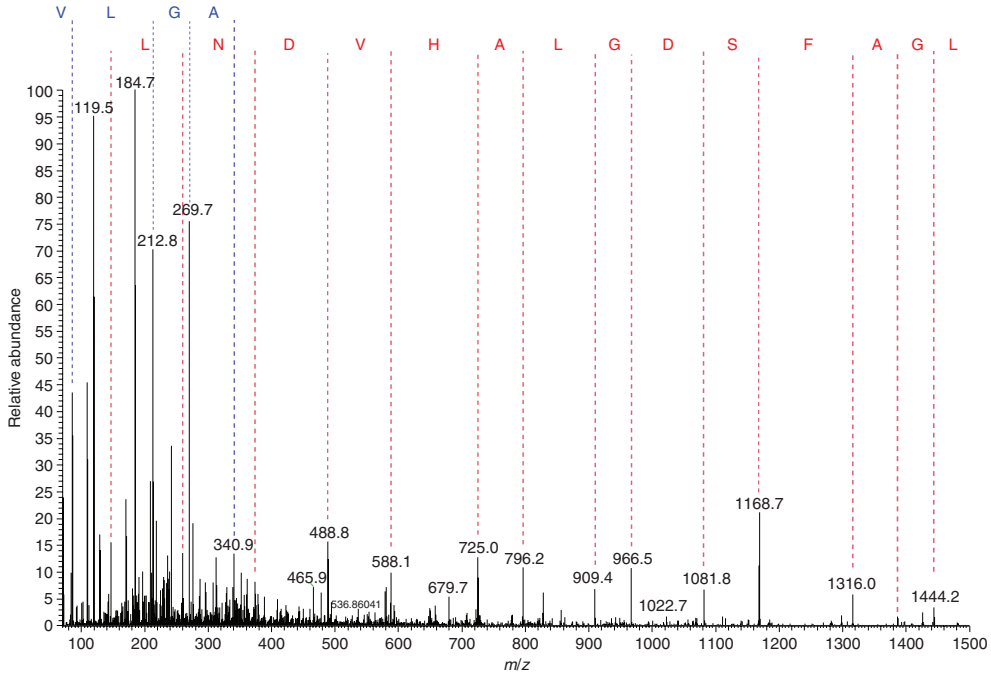

Figure 6: Hb Ullevaal βT9 CID spectrum with annotation of y (red) and b (blue) ions.

a charge difference and probably will be neglected by this approach. Nevertheless, an extra peak in the ion exchange chromatography for determination of glycated Hb led to the suspicion in this case of Hb Ullevaal. However, there are secondary effects possibly caused by conformational changes as a consequence of the mutation. In other cases

post-translational modifications led to the appearance of an interfering fraction while the mutated Hb itself remains cryptic, superimposed by HbA_0.

Several publications have shown the benefit of the additional use of mass spectrometric approaches in the field of Hb analysis [8, 9, 13–16].

The most straightforward approach seems to be MALDI-ToF-MS of the crude Hb mixture. This method has the advantage of being fast and without a lot of sample preparation yielding mass information. On the other hand it has been shown, that even a MS yielding high mass resolution of R = 200,000 does not allow the discrimination of mass differences of 1 or 2 *m/z* whereas a mass difference of 9 *m/z* is clearly distinguishable [8]. Consequently enhancing mass resolution does not necessarily yield unambiguous globin identification. Furthermore, despite the growing use of orbitrap and ToF-MS not in every laboratory a high resolution MS device is available. Even though the globin separation and the mass spectrometry by MALDI-TOF-MS are already studied as separate techniques for the examination of Hb variants [17], still no data are available concerning the combination of globin separation and MS. Looking at single point mutations of the α- and β-globin only for about 40% of the variants charge differences are to be expected (Figure 1). Because of the only use of separation techniques relying on a differing isoelectric point the frequency of Hb variants necessarily are by far underestimated.

In contrast more than 95% of the mutations do show a difference in hydropathicity [8]. Interestingly based on theoretical data all of the variants show at least one of the studied criteria to differ from wild type. Consequently, the use of globin separation in combination with full scan mass spectrometry and ion exchange HPLC allows a screening for Hb variants with a reduced probability to gain false negative results. The example of Hb Ullevaal showed that a comparably small structural difference of one methyl group (Leu→Val) in a 142 amino acid protein causes a rather different chromatographic behavior of the variant globin and can be detected. At the same time the identity of the variants commonly found can be verified more precisely even on protein level (Figure 7). In case the variant globin is separated chromatographically from the wild type entity this also allows a sufficiently precise estimation of the *m/z* without any interference. According to our experience it is possible to detect a mass difference down to 1 *m/z*, as regularly seen in the case of HbE (data not shown).

Our data describe the usefulness of an approach which could be applied on a triple quadrupole MS. Other reports describe the preparative separation of the aberrant Hb or

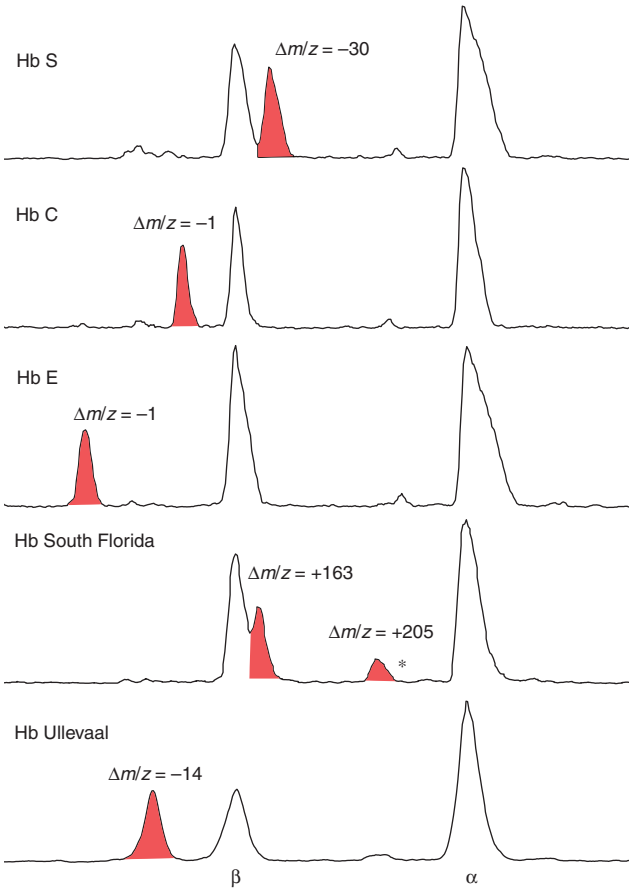

Figure 7: Globin separation of different heterozygous Hb variants. The aberrant globin entity is marked by the red filling and annotated with the *m/z* difference. (*) indicates the acetylated fraction of Hb South Florida.

globin chain prior to the MS analysis or fragmentation experiments. Although this approach is a very thorough basis for further analysis of the variant Hb, for a routine procedure it is too cumbersome [13].

The usefulness of the globin separation is mainly based on differences in hydropathicity though adds a different separation principle. According to our data this parameter contributes a further independent criterion in identification of an Hb variant (Figure 7). However, our experience does not show a good correlation of the theoretically calculated hydropathicity [18] with the retention coefficient of the respective globin. This probably can be explained by secondary effects caused by conformational changes, ion pair effects of TFA, secondary interactions with the column material and others. As a consequence precise estimation of the hydropathicity from the retention behavior is not possible. Furthermore, it cannot completely be excluded, that post-translational modifications or instabilities of respective functional residues cause a

different chemical structure of the phenotype compared to the situation expected from the genetics [19].

Finally with the combination of the mass and retention information of the globin and the tryptic peptides, it is possible to gain fragment spectra containing enough information for the identification of most of the questionable peptides. Furthermore, knowing the affected globin entity provides a basis for possible subsequent targeted genetic analysis.

Basilico et al. describe the use of the peptide map or identification of the mutation bearing peptide by means of the respective m/z and $\Delta m/z$ with subsequent alignment to a variant peptide database using the SEQUEST algorithm as primary strategy [14]. However, this implies to perform a tryptic digest of every sample to be examined and therefore would not be considered as appropriate method for a routine screening of Hb variants.

Although the detection of mutated tryptic peptides by MRM using proteotypic mass transitions is a selective and sensitive approach, it is not possible to use it for the screening of more than 2103 possible single point mutations by technical reason [8].

The approach of screening Hb variants on the protein level can sometimes fail to detect a variant. If the mutation leads to an unstable tryptic peptide, the only hint for the variant present may be a reduced amount of the concerned wildtype peptide. In some cases an amino acid newly introduced by a mutation causes post-translational modifications though more complex and ambiguous results are the consequence. For instance, in the case of Hb South Florida two different new globin entities are found in the globin LC/MS and in the peptide map as well. This is a consequence of a phenomenon that about 20% of the proteins having a methionine as N-terminus are acetylated [20]. In principle higher degrees of oxidation, deamidation and glycosylation as a consequence of a mutation could be considered as well and can result in misleading patterns in the globin separation and subsequent interpretation of the mass difference as well. On the other hand variants with a genetic basis exceeding a single point mutation are likely to be found and characterized by this concept as well.

Variants that are very unstable probably cannot be found in the peripheral red blood cells at all thus can only be discovered by genetic sequencing of the respective globin gene. Furthermore, globins of lower abundance like γ- and δ- globin are hardly to be detected by our routine screening due to the signal-to-noise ratio. The characterization in principle is possible as well if such a low abundance variant is suspected. In so far characterization of both protein and DNA level may be important at least in some cases. Hyperinstable variants can only be detected by genetic sequencing because of lack of the gene product in the peripheral blood.

In the future the possible benefit of a high resolution MS should be evaluated especially concerning the use of de novo sequencing techniques. Another issue to be addressed is the possibility to automate the whole workflow, which is done manually to date. One other question to be answered is, in how far comorbidities with Hb variants called "clinically still" play a role and other diagnostic measures beside HbA_{1c} are influenced [19]. This could be the case, if "clinically still" variants appear in combination with sickle cell anemia were they can have effects in either direction.

Unless the use of mass spectrometry requires trained staff and generates some costs as well, according to our experience the stepwise approach described here is appropriate for routine use, reduces the ambiguity oft the results, helps detecting and identifying the questionable variant and reduces the danger of missing a Hb variant during a screening process.

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